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TITRATION OF HEMAGGLUTINATION-INHIBITING ANTIBODIES TO ECTROMELIA VIRUS BY THE TAKATSY MICROTITRATION METHOD

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Abstract

Combined with the orbital-puncture method for obtaining blood specimens, the Takatsy microtitration method permits repeated serological tests from an individual mouse. The author reports on standardization of the hemagglutination-inhibition reaction by selection of suitable reagents. The method is well suited for large-scale serological diagnosis of ectromelia.

Introduction

Serological tests from mice have been facilitated by the possibility of obtaining greater amounts of serum by the method of orbital puncture described in Ref. 1 and 2. Without killing the animal, a mouse weighing 20 g will furnish a maximum of 0.5 ml of blood. If the puncture method is combined with a microtitration method, repeated tests from the same animal become possible.

In our search for a routine microtitration method of the hemagglutination-inhibiting antibodies to ectromelia, we decided on a modification of the Takatsy titration apparatus. The following reports on investigations for technical standardization of the method by suitable selection and adjustment of the reagents. It would seem that the method is well suited for serological diagnosis of mousepox on a large scale.

Investigative Materials and Methods

1. Microtitration System

We utilized the Takatsy (Ref. 3) microtitration apparatus as modified by Ref. 4. Calibrated loops and pipettes replaced customary pipettes and, instead of individual test tubes, plexiglass plates are utilized which contain cups (8 x 12) with U- and/or V-section.

The series dilutions of mouse sera for the hemagglutination-inhibition reaction (HAHR) were prepared as instructed by Ref. 4. An immune serum accompanied each test. A buffer was added to the first dilution ratio (1:10) as serum control, and the standard antigen dilution to the remaining ratios. The antigen was titrated twice on each plate, both in buffer alone and in 0.05% inactive mouse serum. The dish volumes were equalized to those of the hemagglutination-inhibition reaction by adding the respective dilution fluid.

Subsequently, the erythrocyte suspension was introduced and the plates covered with plastic foil (Permacel, New Brunswick, New Jersey).

It is preferable to perforate the foil over each dish prior to placement in the incubator. We utilized pins with glass heads which were fused into a paraffin block at the same spacing as the centers of the dishes.

Reading was made after placing the plates in the incubator for 30 minutes. Prior to this as well as for each return to the incubator for several readings, the plate content was agitated by horizontal vibration.

The reactions were read by means of a magnifying reflector from below. Before evaluation, we set the plates on edge until complete outflow ["Auslaufen"] of the agglutination-inhibited erythrocytes (about 30 seconds).

2. Evaluation of the Reactions

We utilized the following symbols:

M = membrane: homogenous sediment covers entire bottom of dish;
= positive hemagglutination, negative hemagglutination-inhibition reaction.

\+ = indistinctly outlined sediment containing partial agglutination; does not flow out, flows out incompletely or hesitantly, slides off as a whole.
= partial agglutination, negative or questionable hemagglutination-inhibition reaction.

ϕ = sharply outlined button-like sediment in dish center; flows out ("teardrop").
= negative hemagglutination, positive hemagglutination-inhibition reaction.

3. Testing Loops

In order to test the capacity of the spiral loops, they need only be examined against a light background with a standing loupe. This permits recognition of even very minute air bubbles.

4. Plate Cleaning

Cleaning of the plates is decisive for the reproducibility of the results of titration. After removing the foil in a 0.5% sodium-hypochloride solution, the plates were cleaned with a strong jet of water by using a spray nozzle with holes at the spacing of the dish centers. After actual cleaning as communicated in Ref. 5, the plates were submerged in distilled water of 50°C for 30 minutes and subsequently shaken vigorously. The small residue of water then evaporates quickly.

5. Procuring Blood Specimens

The blood specimens were obtained by puncture of the orbital venous plexus (Ref. 1, 2). Little injury was observed even under repeated puncture of the same orbit.

The pipettes were prepared from glass tubes (internal diameter 2 to 3 mm) over a gas flame. The lumen at the tip should not exceed 0.8 mm at a thin wall thickness. Such a pipette holds about 0.5 ml at a length of 150 mm.

The pipettes were cleaned (Ref. 6) and prepared by annealing with a 1% silicon-oil emulsion ("Bayer-H," 3 hours, 200°C).

6. Serum Preparation

The blood was blown out along the wall into tubes on horizontal supports (Ref. 7). It should not reach the tube bottom. After 60 minutes at room temperature, the tubes were placed vertically and stored for several hours in the refrigerator. The clear serum can then be obtained from the top of the tube. Non-diluted sera were kept in the pipettes described whose lumen was sealed with "Plastillin" and the pipettes stored horizontally in corrugated cardboard at -20°C.

Since the initial dilution of the hemagglutination-inhibition reaction is made with an initial dilution of 1:10, the sera were drawn off with blood-sugar pipettes and prediluted at 1:5 with 0.85% salt solution.

7. Test Erythrocytes

We tested chicken blood (white Leghorn, quail-marked Italians) for its reaction to agglutination by vaccine and cardiolipin flocculation antigen (Ref. 8) and selected the best individuals as donor. The blood was obtained, by means of a hypodermic syringe filled with "Alsever-

solution," from the ulnar vein. The erythrocytes were eluted three times and suspended in Dulbecco phosphate-buffered salt solution. We did not utilize any mixed blood.

8. Preparation of Antigens

We utilized vaccine virus (strain Levaditi; Ref. 7) and ectromelia virus (Elberfeld strain) as hemagglutination-inhibiting antigens which were propagated in HeLa cells and on chorioallantois.

Densely-grown single-layer cell cultures (400-ml flask) were inoculated with 4×10^7 ID_{50}-units of tissue culture in the incubation medium (200 ml). After 24 hours in the incubator, the incubation medium was replaced by the maintenance medium. After 24 hours, part of the cells generally separated spontaneously from the glass surface and the rest was harvested by freezing and thawing.

A mixture of 1,000 g from several flasks was centrifuged at 4°C for 30 minutes and the sediment suspended in McIlvaine-buffered salt solution in 1/10 of the initial volume. In the ice bath, we homogenized the suspension in 8 times for 15 seconds at 24,000 rpm (Ultra-Turrax, Type 18/3, Jahnke & Kunkel Company). The second centrifuge run removed cell debris and the supernatant served as antigen. The latter had a ID_{50} of tissue culture of 10^7 and a hemagglutination titer of 1:90 (vaccine) and/or 1:64 (ectromelia).

Propagation on the egg was made by the technique of Ref. 10.

9. Immune Sera

The immune sera originated from mice surviving intraplantal ectromelia infection who received intraperitoneal injection (three weeks after infection) of 10^6 LD_{50}-units of ectromelia antigen three times at intervals of two weeks each. The sera were tested individually 10 days after the last injection and those of the same titer were combined. Their hemagglutination-inhibition titers were between 1:640 and 1:2,560.

10. Dilution Fluids and Media

0.85% salt solution with variable pH
2% "Tris"-buffered (0.2 M-Tris buffer) salt solution, pH 7.4
McIlvaine-buffered (0.2 M) 2% salt solution, pH 7.1 (Ref. 11)
Dulbecco (PSD) phosphate-buffered salt solution, pH 7.1 (Ref. 9)
Mayer veronal-bicarbonate buffer, pH 7.2 (Ref. 12)

Tissue-culture incubation medium: Hanks salt solution (Ref. 13) with the addition of 0.5% lactalbumin hydrolyzate (Nutritional Biochemical Corporation), 10% inactivated calf serum, and 200 international units penicillin-G + 200 mg/ml streptomycin.

Tissue-culture maintenance medium: same as incubation medium with the addition of Eagle 1% vitamin solution (Ref. 14) and reduction of the calf-serum concentration to 5%.

Discussion of Findings

In attempting to standardize the microtitration method for hemagglutination-inhibition reaction, we investigated several factors including the sensitivity of the reaction in different media, with different antigens and with repeated readings.

Influence of normal serum: vaccine and ectromelia antigens were titrated to normal serum in the form of a checkerboard titration. As expected from the investigations of Ref. 15, serum addition changed the sedimentation picture of the erythrocytes (Ref. 15); M-reactions in buffer medium were converted to +-reactions. This phenomenon vanished with increasing antigen concentration and/or serum dilution.

Plate type: comparative tests in cups with U- and V-section showed the superiority of the latter. With negative reactions, the sediment was concentrated in the cone tip and erythrocyte outflow could be easily read even at low concentration.

Suspension Media for Erythrocytes

The first findings with the microtitration method deviated from those by standard methods (Ref. 15, 16) if we utilized the salt solutions customary with them. The cells appeared shrunk and defective under the microscope.

In the attempt for finding an optimum diultion medium, we tested various salt solutions. We evaluated these from the color, tendency to hemolysis and outflow of the sedimented cells under inclination of the plates. McIlvaine-buffered salt solution and veronal buffer were eliminated because of their agglutinating and/or hemolytic properties and Tris-buffered salt solution because of inhibition of the erythrocyte outflow. Interference through spontaneous agglutination and outflow inhibition resulted from the addition of guinea pig, mouse and calf serum to the salt solution (0.05%).

The Dulbecco buffer (Ref. 9) was shown to be the best of the tested solutions. In relation to the customary salt solution, it also conserves the suspended cells longer.

Erythrocyte Density

Based on the known dependence of the hemagglutination titer on density of the erythrocyte suspension (Ref. 15), we carried out corresponding tests with the microtitration method.

We titrated antigen and immune serum of known valence at the same dilution ratio and parallel to each other on a single plate and added to hemagglutination and/or hemagglutination-inhibition reaction the respective blood-cell suspension and/or antigen dilution + blood-cell suspension (agglutinating system). The reaction volume in hemagglutination was adjusted to that in the hemagglutination-inhibition reaction by addition of the respective suspension fluid. McIlvaine-buffered salt solution and veronal buffer were not further investigated because of their lesser suitability.

The findings are shown in Table 1.

Table 1

Hemagglutinin and Hemagglutination-Inhibition Titers as a Function of Density of the Erythrocyte Suspension in Different Media

Hemagglutination (a)			(c) Erythrozytenkonzentration	(b) Hemagglutinations-Hemmung (Antigen mit 2 agglutinierenden Einheiten, bezogen auf 0,5 %ige Zellsuspension)		
(d) Suspensionsmedium: NaCl 0,85 %	NaCl 0,85 % + 0,05 % Serum	(e) Dulbecco Puffer		NaCl 0,85 %	NaCl 0,85 % + 0,05 % Serum	(e) Dulbecco Puffer
n. a.*	n. a.	n. a.	0,25 %	6	6	6
4**	4	4	0,35 %	6	6	6
5	4	4,5	0,5 %	6	6	6,5
4	4	4	0,7 %	6,5	6,5	6,5
4	4	4	1,0 %	7	7	7
4	4	4	1,4 %	7	7	7,5

* n.a. = not readable.
** titer in log 2 units, initial dilution 1:10.

LEGEND: a - hemagglutination; b - hemagglutination inhibition (antigen with 2 agglutinating units referenced to 0.5% cell suspension; c - erythrocyte concentration; d - suspension media; e - Dulbecco buffer.

Titers are expressed in negative logarithms to the base 2 and referenced to the initial dilution. A + reaction was evaluated arbitrarily as 0.5 log E.

When utilizing a blood-cell suspension of equal to or less than 0.25%, hemagglutination could no longer be read whereas the hemagglutination-inhibition reaction still permitted a unique determination of the terminal

point. The 0.5% solution finally selected for all further tests was used both for hemagglutination and for hemagglutination-inhibition reaction. This density resulted as a compromise between adequate readability and optimum low concentration. If the inhibition titers reached higher values at higher erythrocyte concentration, then this is only an apparent indication of their greater sensitivity, i.e., the antigen was adjusted, with an 0.5% erythrocyte suspension, to two agglutinating units (a.E). After correction of the respective agglutinating system to two agglutinating units, agglutination is more difficult to inhibit at a denser than at a diluted suspension (Ref. 15).

Type of Antigens and Reading

We examined antigens from tissue culture and chorioallantois which furnished the same results. The former are preferable because they are more easily obtained in a large amount, free of hemoglobin, and with low protein admixture.

Comparative titrations of such antigens to ectromelia immune sera are shown in Table 2. With increasing antigen concentration, the sensitivity of the hemagglutination-inhibition reaction to both vaccine and ectromelia antigens is reduced. Moreover, the inhibition titer is a function of the number of readings (shaking of the reaction mixture, sedimentation at 37°C for 30 minutes). Drip turbulence is not sufficient for obtaining the terminal titer. Even a single shaking produces a result which deviates by as much as 0.7 log E from the terminal titer.

Table 2

Hemagglutination-Inhibition Titer as a Function of Number of Readings

(a) Vaccine Antigen: Ablesung nach (Anschütteln, Sedimentieren)	0	30	60	90 Min.	max. Diff.*	(b) Ectromelia-Antigen: 0	30	60	90 Min.	max. Diff.*
(c) Antigenverdünnung (agglutinierende Einheiten)										
16	3,3	3,5	3,0	3,0**	0,5	4,6	4,4	4,2	4,1	0,3
6	5,3	5,5	5,0	5,0	0,5	7,2	6,9	6,9	6,9	0,1
4	6,2	5,8	5,6	5,3	0,6	7,5	6,4	—	6,0	0,4
3	7,7	6,7	6,5	6,0	0,7	8,0	7,4	7,5	6,8	0,7
2	8,0	7,0	7,0	6,8	0,2	8,0	7,9	—	7,4	0,6
1,5	8,0	7,7	7,6	7,2	0,6	8,0	8,0	8,0	7,7	0,3
(d) Antigenkontrolle (agglutinierende Einheiten)	4,0	2,0	1,5	1,0		4,0	2,0	1,5	1,0	

* = maximum titer difference, without consideration of zero reading

** = titer in log 2 units, initial dilution 1:10.

LEGEND: a - vaccine antigen, reading after shaking and sedimentation; b - ectromelia antigen; c - antigen dilution (agglutinating units); d - antigen control (agglutinating units).

The table shows that shaking must be continued until attaining the known antigen titer, here designated as one agglutinating unit. We worked only with non-treated antigens. According to Ref. 16, inactivated antigens -- 30 minutes at 56°C -- furnished titers in the hemagglutination-inhibition reaction which were practically not different from those to non-inactivated antigens.

Antigen Titration

Antigens adjusted on the base of microtitration furnished unsatisfactory results in the hemagglutination-inhibition reaction in the sense that sera titrated against two agglutinating units produced insufficient inhibition titers. Adjustment was therefore made in the form of a checkerboard titration (Table 3). Immune serum from mice was titrated parallel several times and the antigen to be tested, diluted in half-logarithmic steps, was added to every series of serum dilution. The antigen dilution in which the inhibition titer agreed with the one determined previously by standard titration, was utilized in the microtitration test. This made it necessary occasionally to interpolate. Our experiments were carried out with antigens containing two agglutinating units. Although higher inhibition titers were attained with more highly diluted antigens (Table 2), the number of uncertain reactions increased.

Table 3

Block Titration of Vaccine Antigen to Immune Serum from Mice (Inhibition Titer 1:640)

(a) Antigenverdünnung:	1:4	1:6	1:8	1:12	1:16	1:24	1:32	1:48	1:64	1:128
(b) Serumverdünnung										
1:20	+	+	+	+	+	+	+	+	+	+
1:40	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø
1:80	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø
1:160	M	M	+	+	ø	ø	ø	ø	ø	ø
1:320	M	M	M	M	M	M	ø	ø	ø	ø
1:640	M	M	M	M	M	M	M	+	ø	ø
1:1280	M	M	M	M	M	M	M	M	+	ø

(c) Endgültige Antigenverdünnung (interpoliert): 1:56

LEGEND: a - antigen dilution; b - serum dilution; c - terminal antigen dilution (interpolated) = 1:56.

Our investigations have shown that the findings obtained by standard methods on hemagglutination-inhibition reaction with virus of the pox group cannot be directly transposed to the Takatsy microtitration system. However, if its particular features are taken into consideration, it represents an excellent method for large-scale examination of

very small amounts of serum such as occur, for instance, in the diagnosis of ectromelia.

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